Toluene Exposure during the Brain Growth Spurt Reduced Behavioral Responses to Nicotine in Young Adult Rats: A Potential Role for Nicotinic Acetylcholine Receptors in Fetal Solvent Syndrome

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Toluene, an industrial organic solvent, is voluntarily inhaled as a drug of abuse. Toluene has been shown to inhibit the nicotinic acetylcholine receptors. Nicotinic receptors play an important role in brain development during brain growth spurt and early adolescence. The long-term effects of neonatal and adolescent toluene exposure on behavioral responses to nicotine in early adulthood were compared. Sprague-Dawley male and female rats were treated with toluene (500 mg/kg, ip) or corn oil daily over postrnatal day (PN) 4–9 or 25–30. Nicotine-induced hypothermia, antinociception, and seizure activity were examined during PN 56–60. Toluene exposure during the brain growth spurt, but not adolescence, reduced the behavioral responses to nicotine in young adult rats. However, the levels of α4, α7, and β2 nicotinic receptors were not altered in the frontal cortex, striatum, thalamus, hippocampus, and cerebellum by neonatal toluene exposure. These results indicate that toluene exposure during the brain growth spurt produces long-term changes in nicotine sensitivity, which may be unrelated to the total expression levels of α4, α7, and β2 nicotinic receptors. The alterations in nicotine sensitivity may be related to the neurobehavioral disturbance associated with fetal solvent syndrome.

Key Words: toluene; development; nicotinic acetylcholine receptor.

Inhalant abuse has increased dramatically especially in the young over the last decade globally. Survey results consistently show that nearly 20% of children in middle school and high school have experimented with inhaled substances (Anderson and Loomis, 2003). Furthermore, large numbers of inhalant abusers are young women in their childbearing years; therefore, there are rising concerns about the potential negative impact of intentionally inhaled organic solvents on the unborn.

Among organic volatile substances, toluene is highly preferred by abusers because of its euphoric effect, cheapness, and easy availability. One source, resulting in especially high exposure rates of toluene is sniffing glue. Abuse of toluene by pregnant women can lead to an embryopathy also referred to as fetal solvent syndrome. Characteristics of toluene embryopathy include particular craniofacial features, growth retardation, and central nervous system (CNS) dysfunctions, such as microencephaly, brain malformation, and motor and intellectual disability (Pearson et al., 1994). Nevertheless, not all exposed offspring show evident physical features and structural damage. Those who exposed lower doses of toluene might still have important but subtle impairment in synaptic circuitry, reflecting as neurobehavioral disturbance.

The nicotinic acetylcholine receptors (nAChRs) are expressed very early in the nervous system, where they not only are finely regulated during CNS development but probably actively contribute to it. It has been reported that nAChRs are necessary for the normal development of the visual system and sensory cortex, suggesting that adequate nAChR activation in other brain areas during development may be essential for the anatomical and functional maturation of cerebral neuronal circuits (Gotti and Clementi, 2004; Moretti et al., 2004). Experimental evidence indicates that toluene (50μM to 10mM) produces a reversible, concentration-dependent inhibition of acetylcholine-induced current in Xenopus oocytes expressing various nicotinic receptor subtypes and ACh-mediated responses in hippocampal neurons (Bale et al., 2002). In addition, toluene shows potent anticonvulsant effect on nicotine-induced seizures (Chan et al., 2006). It is possible that the inhibitory effect of toluene on nAChRs is associated with the pathophysiology of neurobehavioral disorders related to developmental toluene exposure.

Recent studies have demonstrated that the pathological and behavioral effects of CNS-acting chemicals, such as alcohol, MK-801, and diazepam, on the developing animals depend strongly on the developmental stage during exposure (Ikonomidou et al., 1999, 2000). The period of synaptogenesis, also known as the brain growth spurt, represents the developmental period during which many components of the developing brain are known to be particularly vulnerable to neurotoxicants. The brain growth

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spurt occurs largely during the third trimester of human fetal development, but occurs during the early postnatal period in the rats (Dobbing and Sands, 1979). In addition, the brain undergoes considerable sculpting and remodeling during adolescence. It is also a time of extensive pruning of synapses and of reorganization of many neurotransmitter systems. In a rat model, postnatal days (PN) 28–42 have been conservatively defined as prototypic adolescence based on timing of age-specific behavioral changes, neural changes in brain, puberty, and the growth spurt. However, some harbingers of adolescence may begin in females as early as PN 23 or so (Spear, 2000). In most previous studies on toluene abuse embryopathy, animals were exposed to toluene in gestation (Bowen et al., 2005; Hougaard et al., 1999; Thiel and Chahoud, 1997). This period is prior to the time of brain growth spurt. Therefore, the aim of the present study was to examine whether toluene exposure during brain growth spurt and early adolescence produces persistent dysfunction of nAChRs using several pharmacological and behavioral tests. For that, the nicotine-induced decreases in nicotine-induced receptor subunits in various brain regions were altered in parallel with behavioral changes.

**MATERIALS AND METHODS**

**Materials.** Toluene (HPLC grade, 99.8%) was obtained from Mallinckrodt Baker (Paris, KY). All other chemicals were purchased from Sigma Chemical Co. (St Louis, MO).

**Animal treatment.** Pregnant female Sprague-Dawley rats were supplied from the Laboratory Animal Center of Tzu Chi University (Hualien, Taiwan). Rats were housed individually on a 12/12-h light/dark cycle (lights on 0700 h) at 22°C. All experiments were performed in accordance with the Republic of China animal Protection Law (Chapter III: Scientific Application of Animals) and approved by Review Committee of the Tzu Chi University.

**Toluene exposure during the brain growth spurt.** The day of birth was considered to be PN 0. On PN 4, the litters were culled to 10–12 pups of equal sexes and each litter was randomly assigned to toluene or control group. The toluene animals received 500 mg/kg of toluene (0.1 g/ml dissolved in corn oil, ip) and the control animals received corn oil (0.1 ml/10 g) daily over PN 25–30. Body weight gain was measured over PN 25–30 and behavioral tests were performed on PN 56–60.

**Behavioral tests.** Nicotine hydrogen tartrate was dissolved in saline and injected ip. For both PN 4–9 and PN 25–30 treated animals, the doses of nicotine used in the following behavioral tests were chosen based on the effectiveness in our preliminary studies (data not shown).

**Body temperature.** Rectal temperature was measured by a thermistor probe and digital thermometer (Singa Technology Co., Taipei, Taiwan). Readings were taken just before and after injection of nicotine (3 mg/kg, ip) per 10-min interval for 40 min. The nicotine-induced body temperature change was calculated by the difference in rectal temperature before and after treatment for each rat.

**Hot-plate test.** Rats were placed into a 30-cm–wide Plexiglas cylinder on a hot plate maintained at 52 ± 1°C. The time between placement and jumping or licking its paws was recorded as the index of response latency. Two basal latencies at least 10 min apart were determined for each animal. Then, each animal was injected with nicotine (1 mg/kg, ip) and tested 5, 10, 20, and 30 min after injection. The nicotine-induced antinociceptive response latencies were converted to percentage of maximum possible effect (%MPE), where %MPE = [postdrug latency – baseline]/(cutoff time (40 s) – baseline) × 100.

**Seizure activity.** After injection of nicotine at a dose of 20 mg/kg (ip), each animal was placed in a Plexiglas cage and observed for 30 min. The occurrence of four convulsion signs, which occur in progression, characterizes nicotine-induced seizures: myoclonic twitch (MC) (sudden involuntary muscle jerk), face and forelimb clonus (FF) (rapid writhing movements of the head and neck and/or forelimb clonus), running and bouncing clonus (RB) (whole-body clonus, including running, and jumps), and tonic hindlimb extension (THE) (extreme rigidity, with forelimbs and hindlimbs extended caudally). Results were expressed as percentage of rats expressing the seizure signs.

**Western blotting.** After decapitation, the brains were immediately removed and the brain regions were rapidly dissected out on ice. The samples were homogenized in ice-cold homogenization buffer (50 mM Tris–HCl, 5 mM EDTA, 10 mM EGTA, 0.3% wt/vol β-mercaptoethanol, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride, pH 7.5). The protein concentrations of homogenates were determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). Equal amounts of proteins were boiled for 5 min after dilution with sample buffer. Proteins were separated by electrophoresis through a 10% SDS-polyacrylamide gel and transferred electrophoretically to a nitrocellulose membrane. The NMDA receptor subunits were detected by the amplified alkaline phosphatase Immuno-Blot assay system (Bio-Rad). The membranes were incubated with 5% nonfat dry milk in Tris-buffered saline (TBS: 20 mM Tris, 500 mM NaCl, pH 7.5) containing 0.05% Tween-20 (TTBS). The membranes were then incubated with primary antibody (anti-α4AChR, anti-β2AChR, anti-γ7AChR, anti-GADPH; Santa Cruz Biotechnology, Santa Cruz, CA) at appropriate dilutions in TTBS overnight at room temperature. After the membrane was washed with TTBS, secondary antibody (goat anti-rabbit IgG conjugated with biotin) was added for 1 h. The membranes were washed with TTBS and then incubated with streptavidin-biotinylated alkaline phosphatase complex for 1 h. After extensive washing with TTBS, the immunoreactive bands were visualized using 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium phosphatase.
substrate. Immunoreactive bands were scanned and analyzed with a digital scanning densitometer (personal densitometer, Molecular Dynamics, Sunnyvale, CA). Values for toluene-exposed rats were expressed as values relative to the average of controls, set at 100%.

**Statistical analysis.** Basal body temperature and hot-plate nociceptive response were analyzed by student’s *t*-test. Comparisons between nicotine-induced hypothermia and antinociception at different time points were analyzed by two-way ANOVA with time as the repeated measure followed by Bonferroni posttest. Data for the occurrence of seizure signs were analyzed by chi-square test. A value of *p* < 0.05 was considered statistically significant.

### RESULTS

During the time of toluene exposure (PN 4–9 or PN 25–30), the body weight gain of the toluene-exposed (male: 12.5 ± 1.2 g; 31.8 ± 1.7 g; female: 11.6 ± 1.2; 28.0 ± 1.2) and control rats (male: 12.3 ± 1.3 g, 29.1 ± 2.0 g; female: 11.8 ± 0.7, 28.8 ± 0.5) was similar.

The nicotine-induced hypothermia was measured at PN 56. As shown in Figures 1 and 2, toluene exposure during both developmental periods did not affect the basal rectal temperature in both male and female rats. However, nicotine (3 mg/kg,
ip)-induced hypothermia in neonatal toluene-exposed rats was significantly less severe than control rats in both males and females (male: $F_{1,36} = 14.77, p = 0.002$; female: $F_{1,36} = 8.89$, $p = 0.011$), whereas no significant difference was measured after toluene exposure during adolescence ($F_{1,36} = 0.054, p = 0.82$; female: $F_{1,36} = 0.336, p = 0.573$).

Toluene exposure during the brain growth spurt and adolescence did not affect the nociception in the hot-plate test (Figs. 3A and 4A). Treatment of nicotine (1 mg/kg) produced antinociceptive effect. The antinociceptive effect of nicotine (%MPE) in neonatal toluene-exposed rats was significantly lower than that in control rats after nicotine administration (male: $F_{1,36} = 6.565, p = 0.025$; female: $F_{1,36} = 5.317, p = 0.04$) (Figs. 3B,C). Adolescent toluene exposure did not produce any effect (male: $F_{1,36} = 0.0026, p = 0.96$; Female: $F_{1,36} = 0.0245, p = 0.87$) (Figs. 4B,C).

Four convulsion signs, which occur in progression, MC, FF, RB, and THE, were observed after administration of nicotine (20 mg/kg). As shown in Table 1, the chi-square test revealed that the percentages of neonatal toluene-exposed rats

![FIG. 3. Effects of toluene exposure during the brain growth spurt on basal and nicotine-induced antinociception in the hot-plate test. Animals were treated as described in Fig. 1. Test was performed on PN 58. After three basal trials of hot-plate test (A), nicotine (1 mg/kg, ip) was given. The test was conducted again at 5, 10, 20, and 30 min after nicotine treatment (B, C). Values are mean ± SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$ compared to control levels, according to two-way repeated-measures ANOVA followed by Bonferroni posttest.](https://academic.oup.com/toxsci/article-abstract/101/2/286/1639520)

![FIG. 4. Effects of toluene exposure during adolescence on basal and nicotine-induced antinociception in the hot-plate test. Animals were treated as described in Fig. 2. Test was performed on PN 58. After three basal trials of hot-plate test (A), nicotine (1 mg/kg, ip) was given. The test was conducted again at 5, 10, 20, and 30 min after nicotine treatment (B, C). Values are mean ± SEM ($n = 7$). *$p < 0.05$, **$p < 0.01$ compared to control levels, according to two-way repeated-measures ANOVA followed by Bonferroni posttest.](https://academic.oup.com/toxsci/article-abstract/101/2/286/1639520)
expressing RB and THE were lower than those of control rats after nicotine challenge. Adolescent toluene exposure did not produce significant effect on nicotine-induced seizure susceptibility.

Finally, the neonatal exposure did not affect the levels of α4, β2, and α7 subunits examined in the prefrontal cortex, hippocampus, thalamus, striatum, and cerebellum (Fig. 5).

**DISCUSSION**

The main finding of this study was that toluene exposure during the brain growth spurt (PN 4–9), but not during adolescence (PN 25–30), reduced the hypothermia, antinociception and seizure responses to acute treatment with nicotine in both male and female rats. These results demonstrate that the brain growth spurt is more vulnerable than early adolescence to toluene exposure, reflecting the important principle that timing of exposure defines the insult and outcome. Given the brain growth spurt as a period during which many components of the developing brain are known to be exceedingly sensitive to neurotoxicants, it is possible that toluene exposure during this stage may damage the developing brain in such a way that it permanently alters certain behavioral responses to nicotine.

Although it is generally thought that the adolescence is the most sensitive period to toluene exposure, toluene exposure during different vulnerable-developing stages including gestation, brain growth spurt, and adolescence should be compared in the future. It is well known that nicotine also alters cognition (Levin et al., 2006), anxiety (Picciotto et al., 2002), and arousal (Knott et al., 1998), besides the behavioral responses tested in this study. It is of interest to reveal whether toluene exposure during the brain growth spurt nonspecifically alters all the nicotine-induced behavioral responses.

The mechanisms by which toluene exposure during the brain growth spurt reduced the nicotine-induced hypothermia, antinociception, and seizure responses are unknown. It is possible that the reducing effects of toluene exposure during the brain growth spurt on nicotine-induced behavioral responses are attributed to a reduction in nicotine receptor number or activity after termination of toluene-mediated nicotine antagonism during this critical developmental period. It is well known that nAChRs belong to the large super family of ligand-gated ion channels and are expressed throughout both CNS and peripheral nervous system (PNS), and in nonneuronal cells. Neuronal nAChRs are assembled from only the alpha (α2–9) and beta (β2–4) nicotinic subunits to form both homopentameric (e.g., α7) and heteropentameric (e.g., α4β2, α3β4*) ligand-gating cation channels and are expressed throughout both CNS and PNS (Gotti and Clementi, 2004). The physiological and pharmacological role of different subtypes of nAChRs is distinct. For example, α4 and β2 nAChR subunits, but not α7, are associated with nicotine-induced hypothermia (Tritto et al., 2001, 2004) and antinociception (Damaj et al., 2007), whereas α3 and α7 subtype may underlie nicotine-induced seizures (Adams et al., 2004; Damaj et al., 1999). Even though the nicotine-induced hypothermia and seizures were remarkably insensitive following toluene exposure, the brain growth spurt is the most sensitive period to toluene exposure, reflecting the important principle that timing of exposure defines the insult and outcome.

**TABLE 1**

<table>
<thead>
<tr>
<th>Groups</th>
<th>MC</th>
<th>FF</th>
<th>RB</th>
<th>THE</th>
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</thead>
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<tr>
<td>Toluene exposure at PN 4–9</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 (7/7)</td>
<td>85.7 (6/7)</td>
<td>85.7 (6/7)</td>
<td>28.5 (2/7)</td>
</tr>
<tr>
<td>Toluene</td>
<td>100 (7/7)</td>
<td>100 (7/7)</td>
<td>14.3 (1/7)**</td>
<td>0 (0/7)</td>
</tr>
<tr>
<td>Female rats</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100 (7/7)</td>
<td>100 (7/7)</td>
<td>71.4 (5/7)</td>
<td>42.8 (3/7)</td>
</tr>
<tr>
<td>Toluene</td>
<td>100 (7/7)</td>
<td>100 (7/7)</td>
<td>14.3 (1/7)*</td>
<td>0 (0/7)*</td>
</tr>
<tr>
<td>Toluene exposure at PN 25–30</td>
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<tr>
<td>Male rats</td>
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<tr>
<td>Control</td>
<td>100 (7/7)</td>
<td>100 (7/7)</td>
<td>71.4 (5/7)</td>
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<td>Female rats</td>
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<tr>
<td>Control</td>
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<td>71.4 (5/7)</td>
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<td>100 (7/7)</td>
<td>71.4 (5/7)</td>
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</tbody>
</table>

Note. Animals (seven rats per group) were treated as described in the "Materials and Methods" section and seizure signs were observed after treatment of nicotine (20 mg/kg).

*p < 0.05, **p < 0.01, based on chi-square tests of the difference in occurrence rates between control and toluene-treated rats.
treatment during the brain growth spurt, no changes in the total levels of the α4, β2, and α7 subunits were observed. Other subunits such as α3, α5, and α6 and spinal cord nicotinic receptors not addressed could mediate some of the behavioral actions of nicotine. Alternatively, nAChR activity may be influenced by membrane lipid composition effecting conformational equilibria of nAChR (Baenziger et al., 2000), electrostatic interactions (Meltzer et al., 2006; Song and Pedersen, 2000), enzymatic carboxymethylation (Kloog et al., 1980), conformation changes (Baenziger and Chew, 1997), phosphorylation (Bermudez and Moroni, 2006; Paradiso and Brehm, 1998), endocytosis/exocytosis (Peng et al., 1997), or number of nAChRs trafficking to the cell surface (Christianson and Green, 2004; Pediconi et al., 2004). It needs further investigation to reveal the mechanism contributed to the sensitivity to nicotine by toluene exposure during the brain growth spurt among these possibilities.

It is important to note that nicotine induces changes in various neurotransmitters in many brain regions. The changes are regionally heterogeneous. Nicotine stimulates the release of many different neurotransmitters including glutamate, GABA, acetylcholine, dopamine, norepinephrine, and serotonin by directly activating the nAChRs. The neurotransmitters thus released may participate in the indirect effects of nicotine (Rossi et al., 2005). Any long-term changes occurred in these neurotransmitter systems by neonatal toluene exposure might

**FIG. 5.** Effects of toluene exposure during the brain growth spurt on the levels of nAChR α4, β2, and α7 subunits in the frontal cortex, striatum, thalamus, hippocampus, and cerebellum. Animals were treated as described in Fig. 1, and sacrificed on PN 60; nAChR α4, β2, and α7 subunits were identified in a representative Western blot (A, B). Bar graphs summarized the effects of toluene treatments on the expression of α4 (C), β2 (D), and α7 (E) subunits. Values are mean ± SEM (n = 5).
also contribute to the reduced behavioral responses to nicotine. It has been suggested that the nicotine-induced seizures are related to its enhancement of glutamate release (Damaj et al., 1999). Glutamate release in turn stimulates NMDA receptors (Fedele et al., 1998), thus triggering the cascade of events leading to seizure production. However, toluene exposure during brain growth spurt reduced the seizure sensitivity to nicotine in the present study, whereas the seizure sensitivity to NMDA (Chen and Lee, 2002; Chen et al., 2004) was enhanced with the same treatment protocol in our previous study. It appears that the reducing effects of neonatal toluene exposure on nicotine-induced seizures are specifically attributed to the alterations in nicotinic receptor sensitivity rather than indirectly through changes in NMDA receptors.

In summary, we have found that toluene exposure during the brain growth spurt increased seizure susceptibility to NMDA (Chen and Lee, 2002; Chen et al., 2004) and several GABAA receptor antagonists, such as picrotoxin and bicuculline (Chen and Lee, 2002; Liu et al., 2007), as well as reduced several behavioral responses to NMDA receptor antagonists, such as MK-801 or ketamine (Chen et al., 2004, 2005) and diazepam, an allosteric modulator at GABAA receptors (Liu et al., 2007). The present study presents initial evidence that toluene exposure during the brain growth spurt could disrupt several behavioral responses induced by nicotine. The observed changes related to nicotinic receptors as well as GABAA and NMDA receptors might be implicated in toluene-related neurodevelopmental disorders in humans.

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